Evidence that CTR1-mediated ethylene signal transduction in tomato is encoded by a multigene family whose members display distinct regulatory features

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Abstract

Ethylene governs a range of developmental and response processes in plants. In *Arabidopsis thaliana*, the Raf-like kinase CTR1 acts as a key negative regulator of ethylene responses. While only one gene with CTR1 function apparently exists in *Arabidopsis*, we have isolated a family of *CTR1*-like genes in tomato (*Lycopersicon esculentum*). Based on amino acid alignments and phylogenetic analysis, these tomato *CTR1*-like genes are more similar to *Arabidopsis CTR1* than any other sequences in the *Arabidopsis* genome. Structural analysis reveals considerable conservation in the size and position of the exons between *Arabidopsis* and tomato *CTR1* genomic sequences. Complementation of the *Arabidopsis ctr1-8* mutant with each of the tomato *CTR* genes indicates that they are all capable of functioning as negative regulators of the ethylene pathway. We previously reported that *LeCTR1* expression is up-regulated in response to ethylene. Here, quantitative real-time PCR was carried out to detail expression for *LeCTR1* and the additional *CTR1*-like genes of tomato. Our results indicate that the tomato *CTR1* gene family is differentially regulated at the mRNA level by ethylene and during stages of development marked by increased ethylene biosynthesis, including fruit ripening. The possibility of a multi-gene family of *CTR1*-like genes in other species besides tomato was examined through mining of EST and genomic sequence databases.

Introduction

Ethylene plays important roles in plant growth, development, and physiology including but not limited to impacting seed germination, stem and root elongation, leaf expansion, flower formation, senescence, abscission and fruit ripening (Mattoo and Suttle, 1991; Abeles *et al.*, 1992). Ethylene synthesis can also be induced by, and impact responses to, environmental stresses such as wounding, hypoxia and pathogen attack (Abeles *et al.*, 1992). Economically important fruits such as tomato, apple, pear, melon, squash, peach, avocado, and many other so-called 'climacteric' fruit show increased synthesis and dependence upon ethylene for induction and completion of fruit

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers AY382676 (LeCTR3 cDNA), AY382678 (LeCTR4 cDNA), AY394002 (LeCTR4sv1), AY382679 (LeCTR3 genomic), and AY382677 (LeCTR4 genomic).

ripening. Ethylene has been shown to regulate expression of numerous genes related to ripening (Maunders *et al*., 1987; Lincoln and Fischer, 1988; Zegzouti *et al*., 1999) and thus operates at least in part at the level of gene regulation.

Much of what is known regarding steps in ethylene perception and signal transduction has been realized through studies of the model plant species *Arabidopsis thaliana*. Undoubtedly, one of the most informative mutant screens in *Arabidopsis* for elucidating mechanisms of hormone signal transduction is based upon alteration of the seedling triple response to ethylene. 'Triple response' refers to the morphological changes that seedlings undergo when grown in the dark in the presence of ethylene and include exaggerated apical hook formation, inhibition of root and hypocotyl elongation, and swelling of the hypocotyl (Guzman and Ecker, 1990). This screen has been utilized to identify the majority of plant ethylene signal transduction mutants identified to date (Bleecker *et al*., 1988; Ecker, 1995; Kieber, 1997). The result has been isolation of various components of the signal transduction pathway from ethylene receptors to downstream transcription factors and emergence of an ordered path of gene products involved in ethylene signaling (Ecker, 1995; Chang and Shockey, 1999; Bleecker and Kende, 2000; Stepanova and Ecker, 2000; Alonso *et al*., 2003).

In *Arabidopsis* it has been shown that ethylene is perceived by a family of five ethylene receptors (ETR1, ETR2, ERS1, ERS2, EIN4) with similarity to bacterial two-component histidine kinase sensors (Hua *et al*., 1995; Hua and Meyerowitz, 1998; Sakai *et al*., 1998). Notably, it has been recently demonstrated that an active histidine kinase domain in not required for receptor signal transmission (Wang *et al*., 2003). Ethylene binding to the receptors is mediated through a copper cofactor delivered by the RAN1 protein (Hirayama *et al*., 1999; Rodriguez *et al*., 1999). Ethylene receptors have been divided into two subfamilies based on predicted peptide sequence: subfamily 1 includes ETR1 and ERS1, subfamily 2 includes ETR2, ERS2 and EIN4 (Bleecker, 1999). Double, triple and quadruple mutants in these genes result in constitutive ethylene response phenotypes indicating their function as redundant negative regulators of ethylene signaling (Hua and Meyerowitz, 1998; Wang *et al*., 2003).

Acting downstream of the receptors and possibly as part of a receptor complex (Gao *et al*., 2003) is CTR1, which possesses intrinsic serine/threonine protein kinase activity, and acts as a negative regulator of ethylene responses (Kieber *et al*., 1993; Huang *et al*., 2003). Only one gene with CTR1 function has been isolated to date in *Arabidopsis* and tests for epistasis with available receptor mutants suggest the product of this single gene is involved in signaling from all members of the receptor family (Hua and Meyerowitz, 1998). The N-terminal domain of CTR1 has been shown to associate with subfamily 1 receptors ETR1 and ERS1 and the subfamily 2 receptor ETR2 and has also been shown to be important in the activation of CTR1 (Clark *et al*., 1998; Cancel and Larsen, 2002; Huang *et al*., 2003).

As CTR1 shows high sequence similarity to members of the Raf family of MAPKKKs (Map kinase kinase kinases), it has been speculated that the ethylene signal is propagated through a MAP kinase cascade to downstream targets. It was recently demonstrated that over-expression of SIMKK (an ethylene-inducible *MAPKK*) resulted in a constitutive triple response seedling phenotype and enhanced gene expression of several ethylene-induced genes including *MPK6*, an ethylene-inducible *MAPK* (Ouaked *et al*., 2003). In addition, *MPK6* expression was shown to be constitutively activated in *ctr1* mutants, suggesting a role of this gene in addition to *SIMKK* in ethylene signaling (Ouaked *et al*., 2003).

The model for ethylene signal transduction defined in *Arabidopsis* and the associated gene and mutant resources have permitted comparative genomic and functional analyses in additional species, including important crops where the role of ethylene has important practical consequences. In some instances, the diversity of developmental and response programs may have been facilitated in evolution through modification of ethylene signaling components and/or their regulation. For example, in tomato a number of ethylene signal transduction components homologous to those identified in *Arabidopsis* have been identified and characterized. Six ethylene receptors have been isolated (Wilkinson *et al*., 1995; Zhou *et al*., 1996; Lashbrook *et al*., 1998; Tieman and Klee, 1999; Klee, 2002), five of which have been shown to bind ethylene (Klee, 2002). Three of these are subfamily I receptors (LeETR1, LeETR2, and

NR) while the remainder (LeETR4, LeETR5, and LeETR6) resemble subfamily 2 receptors (Bleecker, 1999). Each tomato receptor gene has a distinct pattern of expression throughout development (including a subset induced during ripening) and in response to external ethylene and pathogens (reviewed by Klee and Tieman, 2002). For instance, *NR* and *LeETR4* gene expression is induced during fleshy fruit ripening (a developmental program non-existent in *Arabidopsis*) and further exhibit functional compensation indicating *in vivo* redundancy (Tieman *et al*., 2000). Three tomato *LeEIL* (*Ein3*-like) genes have also been isolated and were shown to be functionally redundant and to regulate multiple ethylene responses throughout plant development (Tieman *et al*., 2001).

A *CTR1*-like gene (*LeCTR1*) was previously isolated from tomato and shown through complementation of a *ctr1 Arabidopsis* mutant to function in ethylene signaling (LeClercq *et al*., 2002). *LeCTR1* mRNA is up-regulated by ethylene during fruit ripening (Giovannoni *et al*., 1998; Zegzouti *et al*., 1999; LeClercq *et al*., 2002) and, as shown here, is part of a multigene family whose members possess CTR1 function and display differential gene expression. In contrast, in *Arabidopsis* only one *CTR1*-like gene has been implicated in ethylene signaling and its mRNA is constitutively expressed (Kieber *et al*., 1993). We present here experimental evidence of a multigene family of plant *CTR1*-like genes that are able to participate in ethylene signal transduction. The family is differentially regulated by ethylene and stages of development marked by increased ethylene biosynthesis, including fruit ripening. The presence of a multigene family of functional *CTR1* genes is not limited to tomato and the possibility of *CTR1*-like gene loss in *Arabidopsis* was examined. These results suggest that regulation of ethylene signal transduction machinery has been a target for selective pressure.

Materials and methods

Plant material

Arabidopsis thaliana ecotype Columbia plants were grown in a growth chamber under 16 h days at 22 °C. Tomato (*Lycopersicon esculentum* cv. Ailsa Craig) was grown in a naturally illuminated greenhouse under standard conditions.

Isolation of full-length cDNA and genomic clones

An arrayed tomato (cv. Ailsa Craig) callus cDNA library (150,000 primary recombinants) was screened at low stringency with the full-length sequence of *LeCTR1*. Two positive clones with the largest inserts, cLEC056D21 (*LeCTR3*) and cLEC071P14 (*LeCTR4*), were sequenced with an ABI3700 Capillary DNA sequencer and Applied Biosystems BigDye dideoxy terminator reagents (Perkin–Elmer). Two splice variants of *LeCTR4* were recovered and designated *LeCTR4sv1* (cLEC071F7) and *LeCTR4sv2* (pGEMT LeCTR4-sv2#5).

5′ RACE-PCR (Marathon Kit, Clontech) was employed to obtain cDNA spanning the missing 5′-coding sequences of both genes. For *LeCTR3*, the clone obtained through RACE-PCR designated LeCTR3 5′ (2B-1) did not contain the complete coding sequence so an arrayed *Lycopersicon cheesmannii* BAC library (J. Vrebalov and J. Giovannoni, unpublished) was screened with a probe designed from the first 150 bases of LeCTR3 5′ (2B-1). The resulting BAC (LA483 O17H23) was digested with *Hin*dIII and shotgun-cloned into pBluescript (Stratagene). The 5′ end was retrieved via colony lift hybridization to the same probe used to screen the BAC library resulting in identification of LeCTR3 BAC (H1-4). The insert of LeCTR3 BAC (H1-4) was sequenced first with the following primer toward the putative *LeCTR3* start of transcription: TCTR3RevRACE6, 5′-CAAATGACGCCTCCGCATTAGACAAC-3′. Additional primers were designed as new sequence became available until the complete putative coding sequence was obtained. *Pfu* polymerase (Stratagene) was used to PCR the corresponding region from Ailsa Craig genomic DNA with the following primers: TCTR3 BAC H1-4For1, 5′-TCCGATGTGCTTTTTAAGTCAAG-3′ and TCTR3 5′ Rev, 5′-TACTCCCCGGAGA TCGAACTTTCACC-3′. The resulting PCR product was cloned into pGEMT (Promega) to yield a plasmid designated LeCTR3 (Ac+/+Pfu#6) and 3 independent plasmids were sequenced to identify any PCR-induced mutations. LeCTR3 (Ac+/+Pfu#6) extended 513 bases upstream of the predicted start of transcription. Due to difficulties

in cloning the full-length *LeCTR3* RT-PCR product a full-length cDNA sequence was constructed by ligating LeCTR3(Ac+/+Pfu#6) to LeCTR3 5′ (2B-1) with the *Eco*RV internal restriction site found in the overlapping regions (bases 222–228 of LeCTR3 5′ (2B-1)) to create plasmid LeCTR3 (PCR2.1#1).

The full-length cDNA for *LeCTR4* was obtained by performing PCR on callus cDNA with the following primers designed to the predicted sequence ends: TCTR4 5′ For1, 5′-GAAGTTG GGGAACTGAATTTGT-3′ and LeCTR4 3′UTR Rev, 5′-CTTATTTAGCCGCCGAAGAGAAT-3′. The resulting PCR product was cloned into PCR2.1 (Invitrogen) to yield plasmid LeCTR4 (pCR2.1 #8). 3 clones were sequenced to identify any PCR-induced mutation. The full-length cDNA for *LeCTR4sv1* was obtained by cloning the 5′ end obtained from RACE PCR into the 3′-end clone (cLEC071) with the *Nsi*I internal restriction site found in the overlapping regions (bases 130–136 of cLEC071) to yield plasmid LeCTR4sv1 (pBS 2B-2).

To obtain genomic sequence for both *LeCTR3* and *LeCTR4*, an arrayed Ailsa Craig cosmid library (S. Tracy and J. Giovannoni, unpublished) was screened with gel-purified gene-specific 3′-UTR probes for *LeCTR3* and *LeCTR4* (described below). Two cosmid clones for *LeCTR3* (91J17, 153O18) and 4 cosmid clones for *LeCTR4* (28P4, 60O6, 232E16, and 232I8) were subcloned into pBluescript and 19 of the resulting subclones were sequenced with gene-specific primers. Junction regions of the cosmid subclones were sequenced directly from the cosmid to ensure proper assembly of the contigs. Intron/exon boundaries were determined by utilizing the large gap alignment function of the Sequencer program (Gene Codes, Ann Arbor, MI), which allows alignment of cDNA to genomic sequence. Sequences of the cDNA and genomic sequences have been deposited into GenBank (*LeCTR3* cDNA, AY382575; *LeCTR3* genomic, AY382679; *LeCTR4* cDNA, AY382678; *LeCTR4* genomic, AY382677).

Generation of LeCTR *gene-specific probes*

3′-UTR probes were generated by PCR from the corresponding full-length *LeCTR* cDNA sequence with the following primers: LeCTR3 3′UTR For, 5′-TTTCTGCACATATTTGGCA TTC-3′, LeCTR3 3′UTR Rev, 5′-GAACTGTG CATTCCCATTATAAA-3′; LeCTR4 3′UTR For, 5′-CATTTGCACTTGGTATTTGGCTTA-3′; LeCTR4 3′UTR Rev, 5′-CTTATTTAGCCGCC GAAGAGAAT-3′; LeCTR4sv 3′UTR For, 5′-TGTATGATTCCTGCACATCTTTGG-3′; LeCTRsv 3′UTR Rev, 5′-TGGACGAA TTATT GTTGACATACC-3′.

Sequence analysis

Amino acid sequence identities were calculated with the ALIGN program (GeneStream Server, http://www.genestream.org). Amino acid sequence alignments were performed with the CLUSTALX program (Thompson *et al*., 1997). The amino acid sequences for LeCTR3 and LeCTR4 were scanned against the PROSITE database of protein families and domains for predicted patterns and motifs through the ExPASy server (Appel *et al*., 1994). Amino acid sequences were submitted to the PSI-pred (McGuffin *et al*., 2000) program through the ExPASy server in order to predict secondary structure. Phylogenetic trees were constructed with programs from the PHYLIP package (Felsenstein, 1989). Preliminary genomic sequence data for *Brassica oleracea* and *Oryza sativa* as well as EST sequences retrieved from the plant gene indices were obtained from the Institute for Genomic Research (TIGR) website at http://www.tigr.org. All sequences obtained from any of the TIGR databases were reported using the sequence identifier number annotated by TIGR. *AtCTR1* cDNA nucleotide sequence was queried against the database of preliminary *B. oleracea* contigs utilizing the BLASTn function. The AtCTR1 and LeCTR1 N-terminal domain amino acid sequences were queried against the TIGR database of EST collections for each of the plant gene indices available utilizing the tBLASTn function. Sequences that shared at least 50% amino acid identity to either AtCTR1 or LeCTR1 were retained. Sequence IDs were reported as the EST ID if only one EST was identified or as the TC number if more than one EST was identified.

Mapping

Probes for *LeCTR1* (generated by PCR from 800 bp of the promoter region), *LeCTR3* (generated by PCR from the last 1200 bp of *LeCTR3* cDNA)

and *LeCTR4* (3′-UTR probe described above) were surveyed against *Lycopersicon pennellii* and *L. esculentum* genomic DNA digested with 5 different restriction enzymes (*Dra*I, *Eco*RI, *Eco*RV, *Bst*NI, *Hae*III) via DNA gel-blot analysis. After determining which enzyme would provide a useful polymorphism for mapping each gene in a previously developed *L. esculentum*/*L. pennellii* introgression population (Eshed and Zamir, 1994), DNA gel blots with 50–76 *L. esculentum*/*L. pennellii* introgression lines digested with the appropriate enzyme were hybridized with the same *LeCTR* probe used in the initial survey filter to determine to which introgression each locus mapped. *Bst*N1, *Eco*RV, and *Dra*I provided RFLPs for *LeCTR1*, *LeCTR3* and *LeCTR4*, respectively.

Plant transformation

Full-length cDNA sequences for *LeCTR1*, *LeCTR3*, *LeCTR4*, and *LeCTR4sv1* designated LeCTR1 (pGEMT#8), LeCTR3 (PCR2.1#1), LeCTR4 (pCR2.1 #8), and LeCTR4sv1 (pBS 2B-2), respectively, were cloned into the binary plant transformation vector pBI121 (Invitrogen) in the sense orientation and under the control of the CaMV 35S promoter and employing the nopaline synthase (nos) 3′ terminator. The resulting LeCTR1/S, LeCTR3/S, LeCTR4/S and LeCTR4sv1/S constructs were transformed into *A. tumefaciens* strain GV3101 carrying the helper plasmid pMP90. *Arabidopsis ctr1-8* seeds were grown under 12 h day length for 2 weeks, transferred to 16 h day length for 4 weeks and then transformed with the floral dip method (Clough and Bent, 1998). Putative transformants were screened on MS medium containing 50 mg/l kanamycin, 1x Gamborg's vitamins (Sigma), 1% sucrose and 0.7% Phytagar (Gibco) under 16 h of light. Genomic DNA was extracted from each putative transformant and both PCR with CAMV 35S and *LeCTR* gene-specific primers, in addition to Southern analysis with *NPTII* as a probe, were performed to confirm transgene integration and to estimate the number of insertions.

Seedling triple response assay

Arabidopsis seeds were sterilized with 95% ethanol for 1 min followed by 5 min with 50% bleach (2.625% sodium hypochlorite final volume) and re-suspended in 0.1% agarose. Sterilized seeds were plated on sterile cellulose membranes (BioRad) placed on medium containing MS salts, 1x Gamborg's vitamins, 1% sucrose, and 1.2% Phytagar. The plates were incubated at 4 °C in the dark for 4 days and then moved to room temperature and incubated in the vertical position for another 5 days in the dark. Measurements of the hypocotyls and roots were taken for each numbered seedling. The plates were then placed under low light for 2 days and then in 16 h days of high light to allow greening of the cotyledons and true leaf formation. Genomic DNA was extracted from each numbered seedling according to Edwards *et al.* (1991). The pellet was allowed to air-dry and was re-suspended in 10 μl of H_2O; 1 μl was used for a PCR reaction. PCR was performed on each seedling using the 35S forward primer and a *LeCTR* gene-specific reverse primer in order to determine which seedlings were azygous.

RNA isolation

A 2–3 g portion of tissue was ground to a powder with liquid nitrogen by means of a mortar and pestle and extracted with phenol as previously described (Leclercq *et al.*, 2002). The pellet was allowed to air-dry and was re-suspended in DEPC water. The RNA was treated with DNaseI (Promega) followed by phenol-chloroform extraction.

Real-time quantitative PCR

Real-time quantitative PCR was performed with 250 ng total RNA for *LeCTR1*, *LeCTR3*, and *LeCTR4sv*, 350 ng for *LeCTR4*, and 2.5 pg for 18S in a 20 μl reaction volume with Taq-Man One-Step RT-PCR Master Mix reagents (PE Biosystems) on an ABI PRISM 7900HT sequence-detection system. Primer Express software (Applied Biosystems) was used to design gene-specific primers and Taq-Man probes: LeCTR1 forward primer, CATCCTCTTTCTTACTGTGAGAAA ATTTAGA; LeCTR1 reverse primer, CATTTCC CTGTATAAAAACGTTCAGTT; LeCTR1 Taq-Man probe, VIC-CCAACTGCCATTAGCAAT TTTCAGCTCAA-TAMRA; LeCTR3 forward primer, ACTTCAGGCTTTTGTTCCGTACA, LeCTR3 reverse primer, CCACGAGGA AACGTACAAGTCA, LeCTR3 Taq-Man probe, VIC-CAGCCATTTCTCCCAGAAGAGCATTT

GCTAMRA; LeCTR4 forward primer, CAT TTGCACTTGGTATTTGGCTTA; LeCTR4 reverse primer, CTTATTTAGCCGCCGAAGA GAAT; LeCTR4 Taq-Man probe, VIC-CAAAA TCAATCCTGGACAGATGCAGAAACTCAT TAMRA; LeCTR4sv forward primer, CTTG GACCATGTCTGTTTGTGTATC; LeCTR4sv reverse primer, TGGACGAATTATTGTTGA CATACCA; LeCTR4sv Taq-Man probe, VIC-CTGTCTCTTGAATCTAATGAATTTAAGAG CTGTTGCCC-TAMRA; 18S forward primer, CGGAGAGGGAGCCTGAGAA; 18S reverse primer, CCCGTGTTAGGATTGGGTAATTT; 18S Taq-Man probe, 6FAM-CGGCTACCA CATCCAAGGAAGGCA-TAMRA. For LeCTR1, LeCTR3, LeCTR4 and LeCTR4sv, the optimal primer concentration was 900 nM and the optimal probe concentration was 250 nM. Optimal primer and probe concentrations for 18S were 300 and 125 nM, respectively. RT-PCR conditions were as follows: 48 °C for 30 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Samples were run in triplicate on each 384 well plate and were repeated on at least two plates for each experiment. For each sample, a Ct (threshold cycle) value was calculated from the amplification curves by selecting the optimal ΔRn (emission of reporter dye over starting background fluorescence) in the exponential portion of the amplification plot. Relative fold differences were calculated based on the comparative Ct method with 18S as a reference. To demonstrate that the efficiencies of the LeCTR (target) and 18S (reference) were approximately equal, the absolute value of the slope of the log input amount (ng of total RNA) vs. ΔCt was calculated and determined to be <0.1 for each LeCTR and 18S set. To determine relative fold differences, the average Ct value for each target was normalized to the average Ct value for 18S and was calculated relative to a calibrator with the formula $2^{-\Delta\Delta Ct}$.

Results

Cloning of the tomato CTR1 *gene family*

To explore the complexity of *CTR1* sequences in tomato, the *LeCTR1* cDNA (Giovannoni *et al*., 1998) was used to screen an ordered tomato (cv. Ailsa Craig) callus cDNA library (150,000 primary recombinants). This screen resulted in the recovery

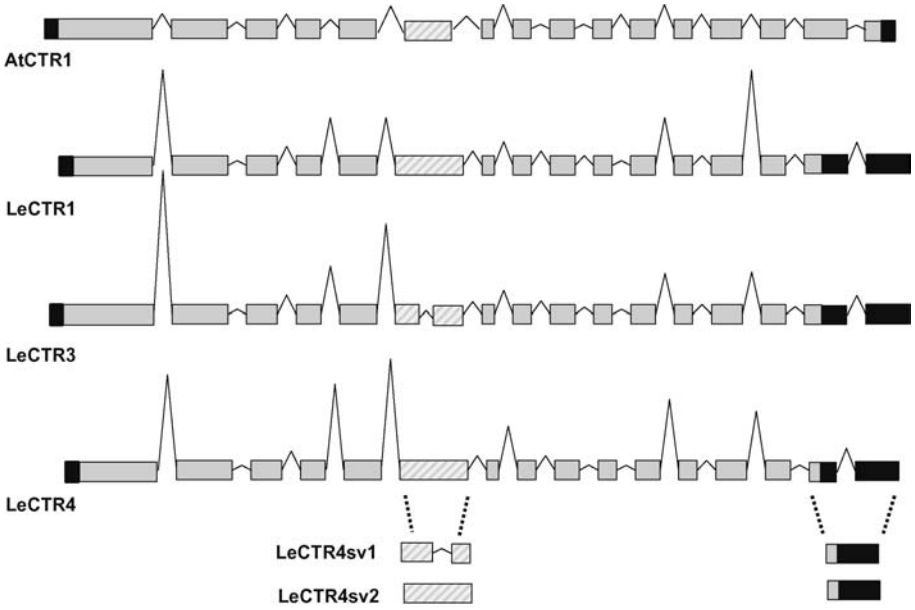

Figure 1. Comparison of the genomic structures of *Arabidopsis CTR1* (L08790) and *LeCTR1* (AY079028) to *LeCTR3* (AY382679) and *LeCTR4* (AY382677). Exons are depicted as boxes and introns as variable sized wedges in proportion to the size of the intron. Regions upstream of the start codon and downstream of the stop codon are represented as black boxes. Exon 6 (with reference to *Arabidopsis*) is shown cross-hatched for each sequence. The dotted lines stemming from *LeCTR4* indicate portions of *LeCTR4* which are differentially spliced in transcripts designated *LeCTR4sv1* and *LeCTR4sv2*.

of two *LeCTR* cDNA sequences similar to, yet distinct from the original *LeCTR1* cDNA and designated *LeCTR3* and *LeCTR4*, as well as additional clones corresponding to *LeCTR1*. Two apparent splice variants of *LeCTR4*, referred to hereafter as *LeCTR4sv1* and *LeCTR4sv2*, were also recovered from this screen. The predicted coding sequences of the *LeCTR4* isoforms vary as a result of differential processing of one exon (Figure 1). Specifically, a stop codon is introduced into the predicted coding sequence as a result of the splicing of the sixth intron in *LeCTR4sv1*. Both *LeCTR4sv1* and *LeCTR4sv2* have identical 3′-UTR sequences and additionally share 67 bp of identical 3′-UTR sequence with *LeCTR4* directly after the predicted stop codon of *LeCTR4*. The *LeCTR4sv1/2* 3′-UTR sequence differs dramatically from *LeCTR4* downstream of this initial 67 bp (222 and 206 bp of 3′-UTR for *LeCTR4* and the splice variants, respectively).

Predicted structural features of tomato CTR1 *proteins*

The *LeCTR3* cDNA contains 3371 bp and translation of the largest open reading frame predicts a protein of 837 amino acids with a molecular mass of 92 kDa. There are 2935 bp in the *LeCTR4* cDNA encoding a predicted protein of 793 amino acids with a molecular mass of 88.5 kDa. LeCTR3 and LeCTR4 share 66% and 70% amino acid identity with the LeCTR1 protein sequence, respectively. Among all four *LeCTR*-like cDNAs identified to date (i.e. those described here and the *AtEDR1*-like *LeCTR2* reported by Lin *et al.*, 1998), LeCTR3 shares the highest percentage of amino acid identity with AtCTR1 in both the N-terminal (variable) and conserved C-terminal protein kinase domains (Table 1). Within their respective kinase domains, LeCTR1, LeCTR2, LeCTR3 and LeCTR4 have a protein kinase ATP-binding site signature (IGAGSFGTVH) found in all protein kinases (Schenk and Snaar-Jagalska, 1999) as well as a serine/threonine protein kinase active site signature (IVHRDLKSPNLLV) found in serine/threonine kinases including Raf and AtCTR1 (Kieber *et al.*, 1993). The 11 subdomains common to all known protein kinases (Hanks and Quinn, 1991; Hanks *et al.*, 1988) are also perfectly conserved in LeCTR1, LeCTR2, LeCTR3 and LeCTR4. All of these aforementioned domains are conserved in the LeCTR4 splice variant, LeCTR4sv2. However, the stop codon in the LeCTR4sv1 predicted 488 amino acid peptide sequence occurs just before the kinase domain, thus the kinase domain would not exist in this isoform if it is successfully translated. The N-terminal domain of the predicted tomato and *Arabidopsis* CTR1 proteins, though more variable (Table 1), also possess a number of interesting structural features conserved to varying degrees among the various sequences. For example, LeCTR3 has an ATP/GTP binding site motif A (P-loop; [AG]-x(4)-G-K-[ST]) at amino acid residues 49–56 and proposed to be involved in binding ATP or GTP in Ras and other proteins (Saraste *et al.*, 1990). This motif is also found in AtCTR1 but not in LeCTR1, LeCTR2 or LeCTR4. Additionally, LeCTR1, LeCTR2, LeCTR3, LeCTR4 and the LeCTR4 splice variants demonstrate conservation of the CN box, found in the N-terminal domain of AtCTR1 and other proteins with domains showing high homology to the CTR1 kinase domain (Huang *et al.*, 2003).

AtCTR1 is one of six *Arabidopsis* MAPKKKs belonging to subclass B3 of group B MAPKKKs, which are related to the Raf kinases and have extended N-terminal domains (Ichimura *et al.*, 2002). Surprisingly, phylogenetic analysis of the four LeCTR predicted peptide sequences, the six *Arabidopsis* sequences and several homologues from rice, barley and rose, indicated that *AtCTR1* is more similar to *LeCTR1*, *LeCTR3* and *LeCTR4* than to any of the other five members of the

Table 1. Amino acid identity (%) between each of the four *LeCTR* cDNAs and AtCTR1 in the N-terminal domain, kinase domain and the full ORF.

AtCTR1	LeCTR1	LeCTR2	LeCTR3	LeCTR4
N-terminal domain	50.0	22.0	57.4	48.9
Kinase domain	84.0	59.5	87.7	83.4
Full ORF	60.7	32.4	67.2	59.6

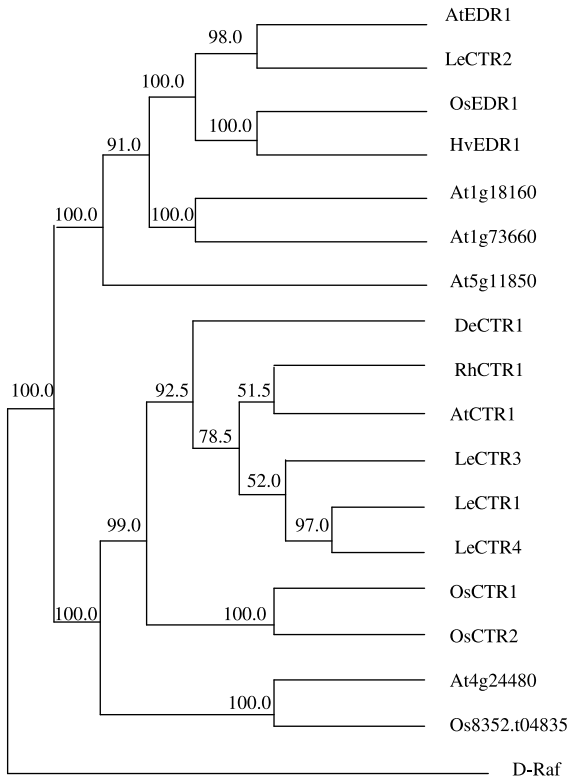

Figure 2. Phylogenetic analysis of tomato (Le), *Arabidopsis* (At), barley (Hv), rice (Os), *Delphinium* (De), and rose (Rh) reported and putative MAPKKKs. Full-length amino acid sequences were aligned with ClustalX. The phylogenetic tree was constructed with programs from the Phylip package: the Seqboot program was used to generate a set of 100 bootstrapped sequence alignments, 100 bootstrapped trees were generated with ProtPars and then Consense was used to choose a consensus tree. D-Raf (*Drosophila* Raf) was used as an outgroup. The numbers at the forks indicate the number of times the group consisting of the species which are to the right of that fork occurred among the trees, out of 100 trees.

Arabidopsis MAPKKK subfamily (Figure 2). Based on amino acid identity and phylogenetic analysis, *LeCTR2* appeared to be more similar to *AtEDR1*, a *MAPKKK* involved in plant defense response, than the other *LeCTR* genes as was previously reported (Frye *et al*., 2001).

Structure of the LeCTR *gene family*

The genomic structure of *LeCTR1* shares with *AtCTR1* conservation of the number, size and position of exons (LeClercq *et al*., 2002). To determine if this conservation in genomic structure was also preserved in *LeCTR3* and *LeCTR4*, genomic sequence information was obtained through screening an arrayed tomato genomic cosmid library with gel-purified gene-specific 3′-UTR probes. Structural analysis revealed that, similar to *LeCTR1* and *AtCTR1*, the *LeCTR4*-coding sequence consisted of 15 exons interrupted by 14 introns while the *LeCTR3*-coding sequence contained 16 exons and 15 introns (Figure 1). In most cases, the size of the introns remained conserved between the members of the *LeCTR* family though with several notable exceptions. For example, intron 1 ranges from 2.18 kb (*LeCTR4*) to 5.7 kb (*LeCTR3*). Intron size was not conserved between the tomato and *Arabidopsis CTR1* genomic sequence, and was generally larger in tomato. In contrast, the size and position of exons was conserved between *AtCTR1* and all of the tomato *CTR*s with the exception of the number of amino acids in the first and last exons in addition to an intron in some versions of exon 6 (Figure 1). Genomic sequences for both *LeCTR3* and *LeCTR4sv1* contain an intron that interrupts exon 6. The intron in both *LeCTR3* and *LeCTR4sv1* occurs in a region of the coding sequence after the CN domain and just before the start of the kinase domain where there is little conservation in amino acid sequence among all the *CTR*s (Figure 3), suggesting a region whose function may be primarily to join adjacent domains. Amino acid sequences were examined for predicted secondary structure (see Materials and methods) and no obvious changes were predicted as a result of the lack or addition of the exon 6 intron sequence into the ORF.

LeCTR1, *LeCTR3* and *LeCTR4* genes have been placed on the tomato introgression line map developed by Eshed and Zamir (1994). The *LeCTR1* and *LeCTR4* loci both map to introgressions 10-2 and 10-3 on chromosome 10, which along with their homology may be suggestive of a gene duplication event, while *LeCTR3* maps to introgression 9-1-3 on chromosome 9. None of these loci are linked to the tomato *Epi* locus (on chromosome 4) that when mutated results in seedling, leaf and root phenotypes consistent with those anticipated for a CTR1 mutation (Barry *et al*., 2001).

Complementation of Arabidopsis CTR1 *mutants*

To determine whether *LeCTR* genes indeed encoded MAPKKKs involved in ethylene signal

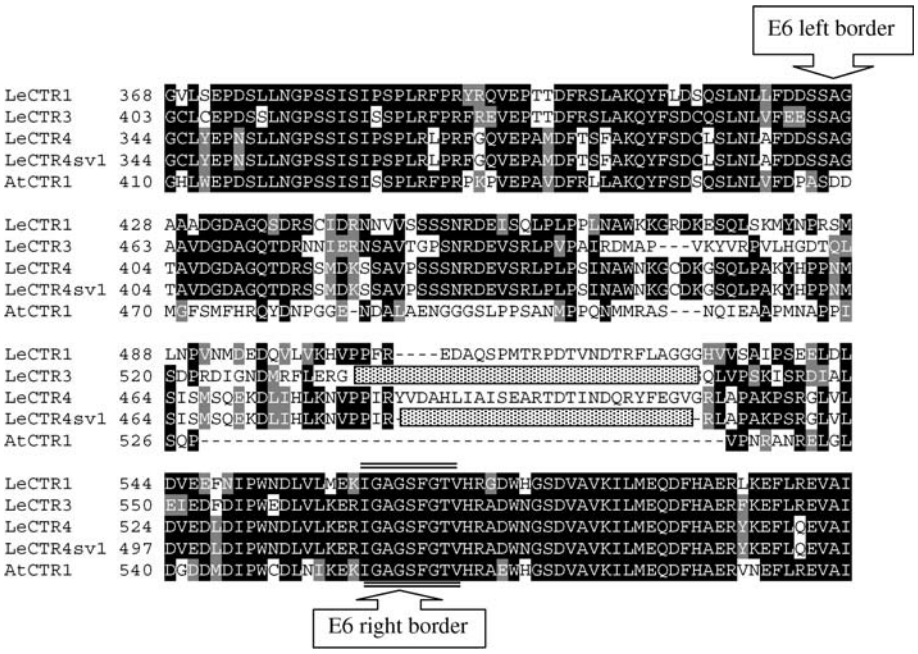

Figure 3. Amino acid alignments of AtCTR1, LeCTR1, LeCTR3, LeCTR4 and LeCTR4sv1 spanning exon 6. Identities between proteins are indicated by shaded squares. The left and right borders of exon 6 are indicated by arrows. The large gray rectangles depict where introns exist in *LeCTR3* and *LeCTR4sv1* genomic sequence that are spliced out in the coding sequence. The first subdomain of the kinase domain is marked above and below with a double line.

transduction, constructs expressing each gene were transferred into *Arabidopsis ctr1* mutant genotypes to assay their respective abilities to complement loss of AtCTR1 function. LeCTR1 has been previously shown capable of complementing the constitutive triple response phenotype of the *Arabidopsis ctr1-1* mutant (Leclercq *et al*., 2002). *ctr1-1* harbors a mutation disrupting the kinase activity of CTR1 (Huang *et al*., 2003). To determine whether or not additional tomato *CTR1*-like genes also encode ethylene signaling CTR1 functions, we delivered constructs expressing LeCTR1, LeCTR3, LeCTR4 or LeCTR4sv1 cDNA in the sense orientation via the CaMV 35S promoter. The *ctr1-8* mutant was selected over *ctr1-1* in part because *ctr1-8* proved more amenable to transformation due to sterility problems with *ctr1-1*.

The ability of the constructs to complement the constitutive triple response and reduced adult plant size phenotypes of *ctr1-8* was assayed. When seedlings were grown in the dark for 5 days, LeCTR3 could fully restore the inhibited hypocotyl length and root length of the *ctr1-8* mutant to wild-type (Figure 4A, B). LeCTR1 and LeCTR4 were not able to restore inhibited hypocotyl length but did partially restore root length. LeCTR4sv1 was unable to complement either hypocotyl or root length in *ctr1-8* (Figure 4A, B). Adult rosette and inflorescence size could be fully restored to wild-type by LeCTR3 and LeCTR4 and was partially recovered by LeCTR1, but not by LeCTR4sv1 (Figures 5 and 6).

Expression analysis of CTR *genes*

A quantitative RT-PCR expression profile for *LeCTR1* was reported previously (LeClerq *et al*., 2002) and was included here for comparison to *LeCTR3* and *LeCTR4* as all were originally performed simultaneously (Figure 7). *LeCTR4* could be distinguished from the two *LeCTR4* splice variants *LeCTR4sv1* and *LeCTR4sv2* (which were not distinguished from each other in this assay) due to the fact that both splice variants share a 3′-UTR sequence distinct from *LeCTR4,* and thus employed as the target for expression monitoring. All messages were shown to be of relatively low abundance based on difficulty of detection via RNA gel-blot analysis (data not shown).

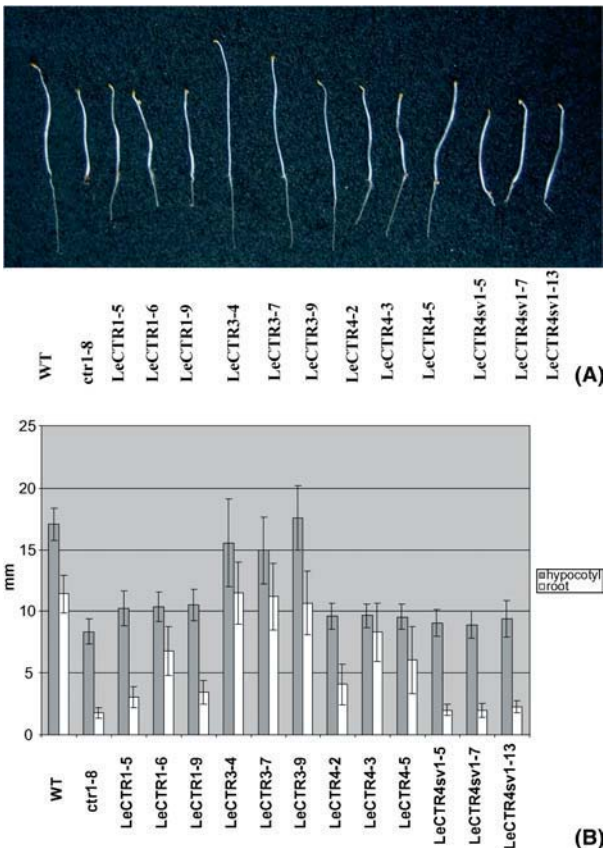

Figure 4. (A) Phenotypes of 5-day old etiolated *Arabidopsis* seedlings in transgenic lines over-expressing a specific LeCTR cDNA in the *ctr1-8* mutant background compared to the *ctr1-8* mutant and wild type. Three independent trangenic lines are pictured from left to right: LeCTR1 (1-5, 1-6, 1-9), LeCTR3 (3-4, 3-7, 3-9), LeCTR4 (4-2, 4-3, 4-5) or LeCTR4sv1 (4sv1-5, 4sv1-7, 4sv1-13). (B) Hypocotyl and root length of the etiolated seedlings. Each histogram represents the mean of measurements taken on 30 seedlings and the vertical bars indicate the confidence interval.

LeCTR3, *LeCTR4* and *LeCTR4sv* accumulated to higher levels in leaves than fruit, which remained low for all three RNAs throughout fruit ripening. In contrast, *LeCTR1* transcript increased markedly coincident with the onset of ripening (Figure 7). During flower development, levels of all three *LeCTR* transcripts decreased 1–3-fold during anthesis compared to the levels observed in unopened buds. While there was a 1–2-fold increase in levels of *LeCTR3*, *LeCTR4* and *LeCTR4sv* in flowers undergoing senescence as compared to anthesis, clearly more pronounced is the 5-fold increase in *LeCTR1* transcript during that same developmental interval. In addition, *LeCTR1* transcripts were 5-fold higher in abscission zones harvested from pedicels of flowers at anthesis stage than in the corresponding flowers. No such abscission-related increase in transcript accumulation was observed for the *LeCTR3*, *LeCTR4* or *LeCTR4sv* transcripts (Figure 7). In summary, *LeCTR1* induction is associated with tissues at stages of development associated with increased ethylene (fruit ripening, pedicel abscission, petal senescence) as reported previously (LeClercq *et al.*, 2002) while *LeCTR3* and *LeCTR4* transcripts are not.

It has been reported that *AtCTR1* is not inducible by ethylene in seedlings (Kieber *et al.*, 1993; Gao *et al.*, 2003), however, a more comprehensive analysis of the ethylene inducibility of *AtCTR1* that could address whether or not this is a tissue-specific phenomenon has not been published. Consequently we examined *AtCTR1* message levels in leaves, stems and siliques from adult plants treated with and without 50 ppm ethylene for 24 h and determined that *AtCTR1* is not in-

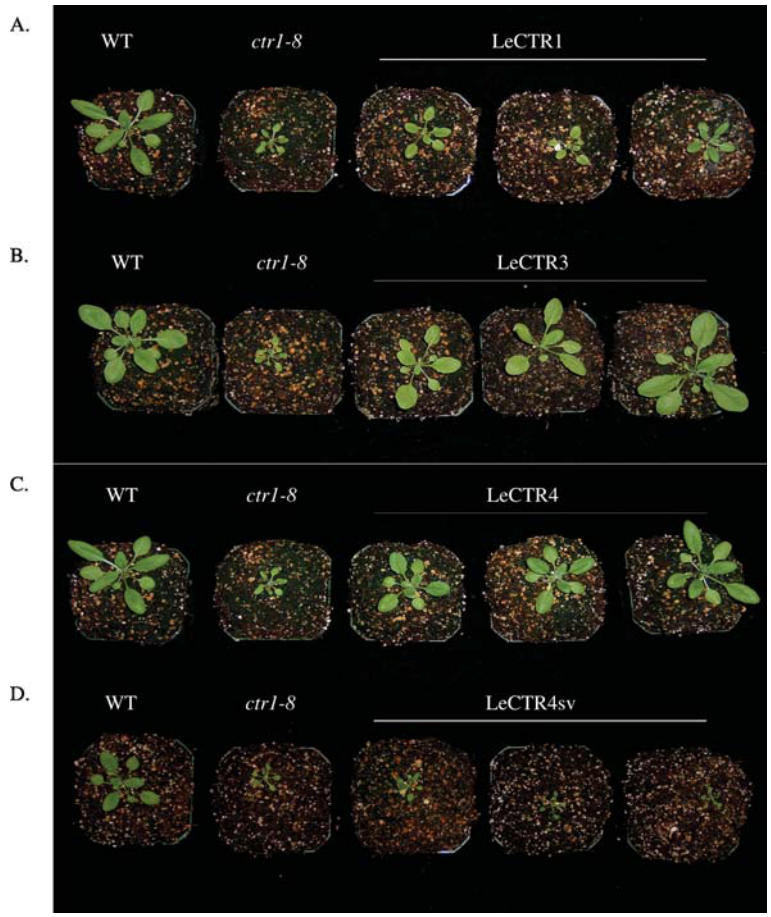

Figure 5. *Arabidopsis* rosette stage phenotypes of the transgenic lines over-expressing a specific LeCTR gene in the *ctr1-8* mutant background compared with that of wild type and the *ctr1-8* mutant. Pictured in each panel from left to right are WT, *ctr1-8* and three independent lines for each *LeCTR* gene. (A) *LeCTR1*-over-expressing lines (1-5, 1-6, 1-9). (B) *LeCTR3*-over-expressing lines (3-4, 3-7, 3-9). (C) *LeCTR4*-over-expressing lines (4-2, 4-3, 4-5). (D) *LeCTR4sv1*-over-expressing lines (4sv1-5, 4sv1-7, 4sv1-13).

duced by ethylene in these tissues under these experimental conditions (data not shown).

We have previously reported that *LeCTR1* is ethylene-inducible in mature green fruit, leaves and roots of tomato (LeClercq *et al.*, 2002). A time course of mature green fruit treated with ethylene was generated to more fully characterize the dynamics of ethylene responsiveness of all the *LeCTR* transcripts (Figure 8A). While *LeCTR1* responded relatively rapidly to ethylene, maintaining elevated levels throughout the 24 h time course, the other *LeCTR* messages failed to accumulate above levels observed in untreated mature green fruit at any point throughout the experiment. Along the same lines, *LeCTR3*, *LeCTR4* and *LeCTR4sv* did not demonstrate significant accumulation in response to ethylene (as did *LeCTR1*) in either leaves or roots (Figure 8B).

Evidence for a CTR *gene family in other species*

There are extensive similarities in genome structure and sequence found among members of the corresponding families to which *Arabidopsis* and tomato belong (*Brassicaceae* and *Solanaceae*, respectively) facilitating a sequence based homology approach for determining the existence of multiple *CTR1*-like genes in *Brassicaceae* and *Solanaceae*. *AtCTR1* cDNA nucleotide sequence was queried against the database of preliminary *B. oleracea* genomic sequence contigs (http://www.tigr.org/tdb/e2k1/bog1). Two sequences were retrieved which spanned the corresponding region

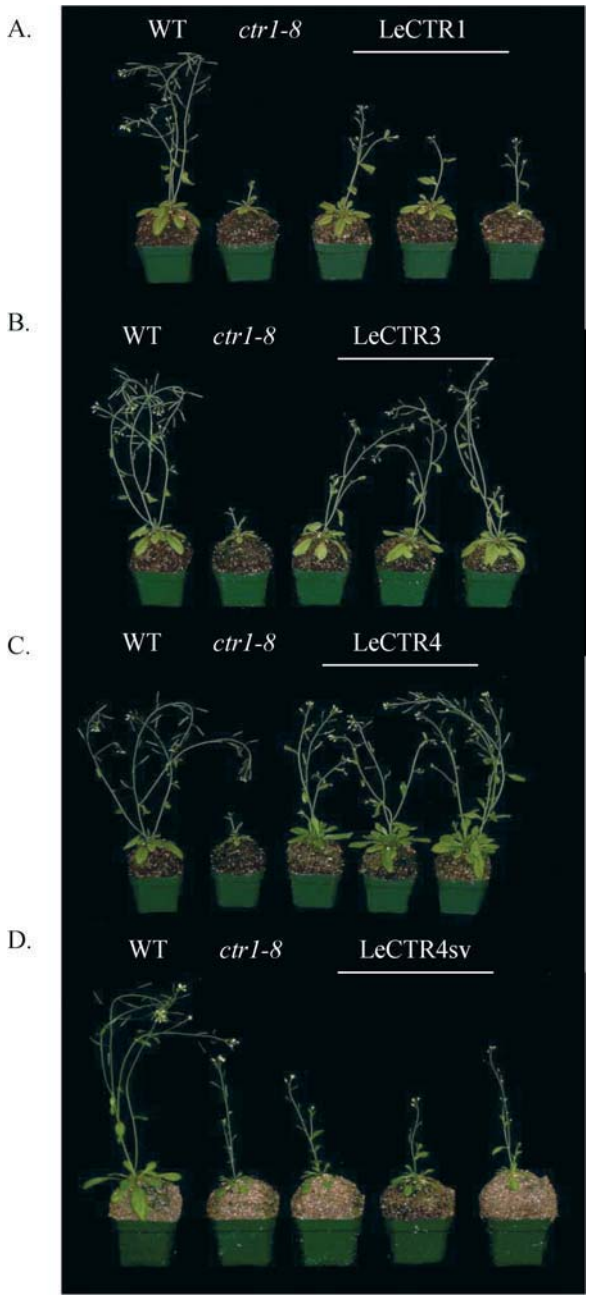

Figure 6. Flowering-stage phenotypes of the transgenic lines over-expressing a specific *LeCTR* gene in the *ctr1-8* mutant background compared with that of wild type (WT) and the *ctr1-8* mutant. Pictured in each panel from left to right are WT, *ctr1-8* and three independent lines for each *LeCTR* gene. (A) *LeCTR1*-over-expressing lines (1-5, 1-6, 1-9). (B) *LeCTR3*-over-expressing lines (3-4, 3-7, 3-9). (C) *LeCTR4*-overexpressing lines (4-2, 4-3, 4-5). (D) *LeCTR4sv1*-over-expressing lines (4sv1-5, 4sv1-7, 4sv1-73). The plants in panel D were photographed at a later stage to indicate there was still no complementation at even later stages of plant development.

of exon 2 in *Arabidopsis* sharing 92% nucleotide identity to each other and 91% nucleotide identity to *AtCTR1*, indicating the presence of multiple copies of *CTR1* in *B. oleracea* (Table 2). These two sequences share only 41–42% amino acid identity to *At4g24480* which is the next most similar sequence to *AtCTR1* in the *Arabidopsis* genome, providing further evidence that the two sequences

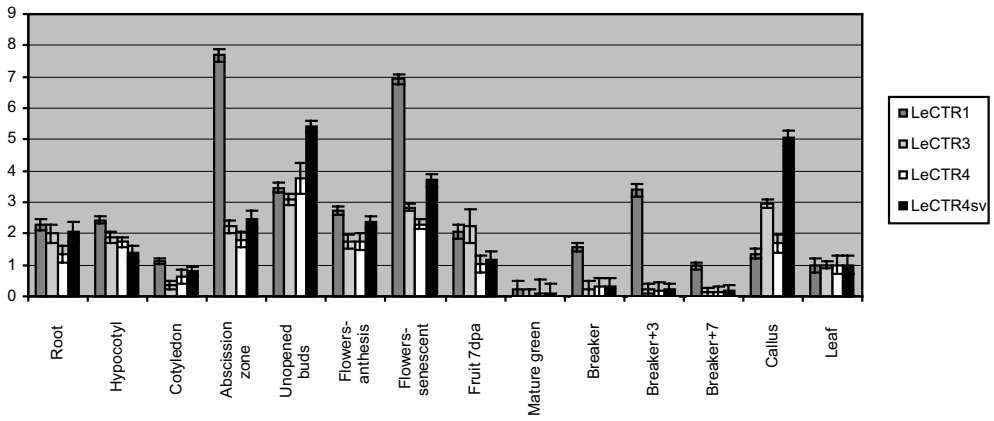

Figure 7. Differential expression of the *LeCTR* gene family. RNA was extracted from different tissues at indicated stages of development and *LeCTR1*, *LeCTR3*, *LeCTR4* and *LeCTR4sv* transcript levels were assessed by real-time quantitative PCR. The *y* axis refers to the fold difference (ΔΔCt) in a particular *LeCTR* message level relative to its level found in leaf.

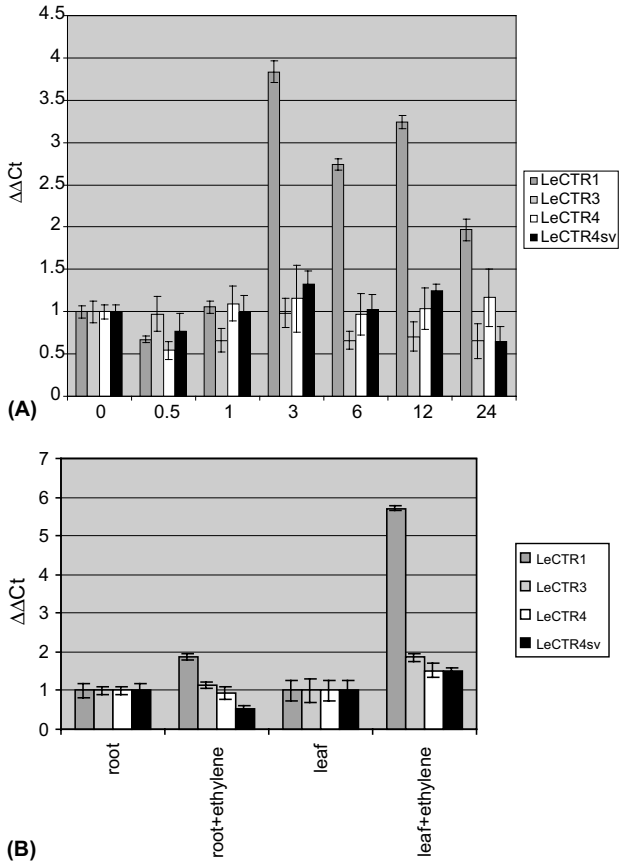

Figure 8. Ethylene inducibility of tomato *CTR1*-like transcripts. (A) Mature green fruit were treated with 20 ppm ethylene for lengths of time ranging from 0.5 to 24 h. RNA was extracted from the fruit and real-time quantitative RT-PCR was performed to determine relative fold differences in gene expression for *LeCTR1*, *LeCTR3*, *LeCTR4* and *LeCTRsv*. ΔΔCt on the *y* axis refers to the fold difference in a particular *LeCTR* message level relative to its level found in the untreated control. (B) Six-week old plants were placed in a sealed chamber and gassed with air or 20 ppm ethylene for 8 h. RNA was extracted from the tissues and real-time quantitative RT-PCR was performed. ΔΔCt on the *y* axis refers to the fold difference in a particular *LeCTR* message level relative to its level found in air-treated root and leaf, respectively.

Table 2. Putative *CTR1*-like sequences obtained from TIGR genome and EST database searches (Materials and methods).

B. oleracea	BOGAC87TR, BOHCQ46TR
G. arboreum	BF274343
G. max	TC193259, BQ611508
H. annuus	BU026195
L. sativa	TC5349, BU008750
M. truncatula	TC93812, TC81131
O. sativa	OsCTR1 (TC136191) (8351.t030726)
	OsCTR2 (CB626810) (8352.t04853)
S. bicolor	CD229655
S. tuberosum	BE919922, BE342235, TC72396
T. aestivum	BJ315794
Z. mays	TC203507

retrieved were in fact both more similar to *AtCTR1* than any other sequence in the *Arabidopsis* genome. In an effort to identify *CTR1*-like genes in the *Solanaceae*, each *LeCTR* cDNA was queried against the TIGR potato EST collection (www.tigr.org) and two single ESTs and one contig were identified (Table 2). One of the singletons (BE919922) does not overlap the other two sequences, thus it is possible that it does not represent a distinct gene. Nevertheless, each sequence corresponded to a different *LeCTR* with 94–98% nucleotide identity, indicating the existence of a *CTR1* multigene family in potato (data not shown).

To identify *CTR1* multi-gene families in other plant species, we submitted both the *AtCTR1* and *LeCTR1* N-terminal domain amino acid sequences into the TIGR database of EST collections for each of the plant gene indices available. We retrieved 13 putative *CTR* sequences from 9 different species (Table 2). All of these sequences contained conservation in the CN domain and those sequences that extended just downstream of the CN domain show additional conservation, which based on our analysis appears to be specific to *CTR*-like genes involved in ethylene signaling (i.e. not in *LeCTR2* or *AtEDR1*) (Figure 9). We have designated the region the EC (ethylene CTR) domain. Because of the ca. 3 kb transcript length of *CTR* genes, some were likely missed due to incomplete cDNA synthesis in EST library construction. The kinase domain could not be used for comparative analysis due to the overwhelming number of non-*CTR* kinases that were returned (data not shown).

Multiple *CTR1*-like sequences were obtained for lettuce, soybean, *Medicago*, and rice. Of most interest were one EST contig (TC136191) and one EST singleton (CB626819) retrieved from the rice EST collection that share 65.8–71% amino acid identity to *AtCTR1* in the CN domain while only 51.2% and 58.8% identity to *At4g24480*. The TC136191 and CB626819 sequences were queried against the rice genomic sequence database (http://www.tigr.org/tdb/-e2k1/osa1/) in order to obtain putative full-length protein sequences for both genes. The TC136191 and CB626819 EST sequences corresponded to 8351.t03037 and 8357.t03295 predicted protein sequences, respectively. A third putative *CTR1*-like rice gene (8352.t04835) was also identified during this search. A phylogenetic tree was constructed using the full-length protein sequences of the putative *CTR1*-like clones from rice in order to determine if they were more similar to reported and putative *CTR1*-like genes or other subgroup B3 MAPKKK genes (Figure 2). Both 8351.t03037 and 8357.t03295 were more similar to *CTR*-like genes than an other MAPKKKs, while 8352.t04835 was more similar to At4g24480. We designated the rice gene represented by 8351.t03037, *OsCTR1*, and that represented by 8357.t03295, *OsCTR2*. Interestingly, OsCTR1 and OsCTR2 show conservation of both the CN domain and the EC domain while Os8352.t04835 only shares conservation in the CN domain (Figure 9). All three sequences contain signatures described earlier that are important for serine/threonine kinase activity.

As *At4g24480* is the gene most similar in sequence to *AtCTR1* in the *Arabidopsis* genome, it might be a likely candidate to exhibit CTR1 function. However, two homozygous lines obtained from SALK containing verified T-DNA inserts in the *At4g24480* did not display constitutive ethylene response in etiolated seedlings or in the adult plants (data not shown). Furthermore, EDR1, which is also a member of this MAPKKK family, has been implicated in the negative regulation of defense responses in plants and does not exhibit any CTR1-like phenotypes indicating it probably functions in a pathway separate from the ethylene-response pathway (Frye *et al.*, 2001). Together, these results provide supporting (though not conclusive) evidence that CTR function is most likely encoded by only one *CTR1* gene in *Arabidopsis*.

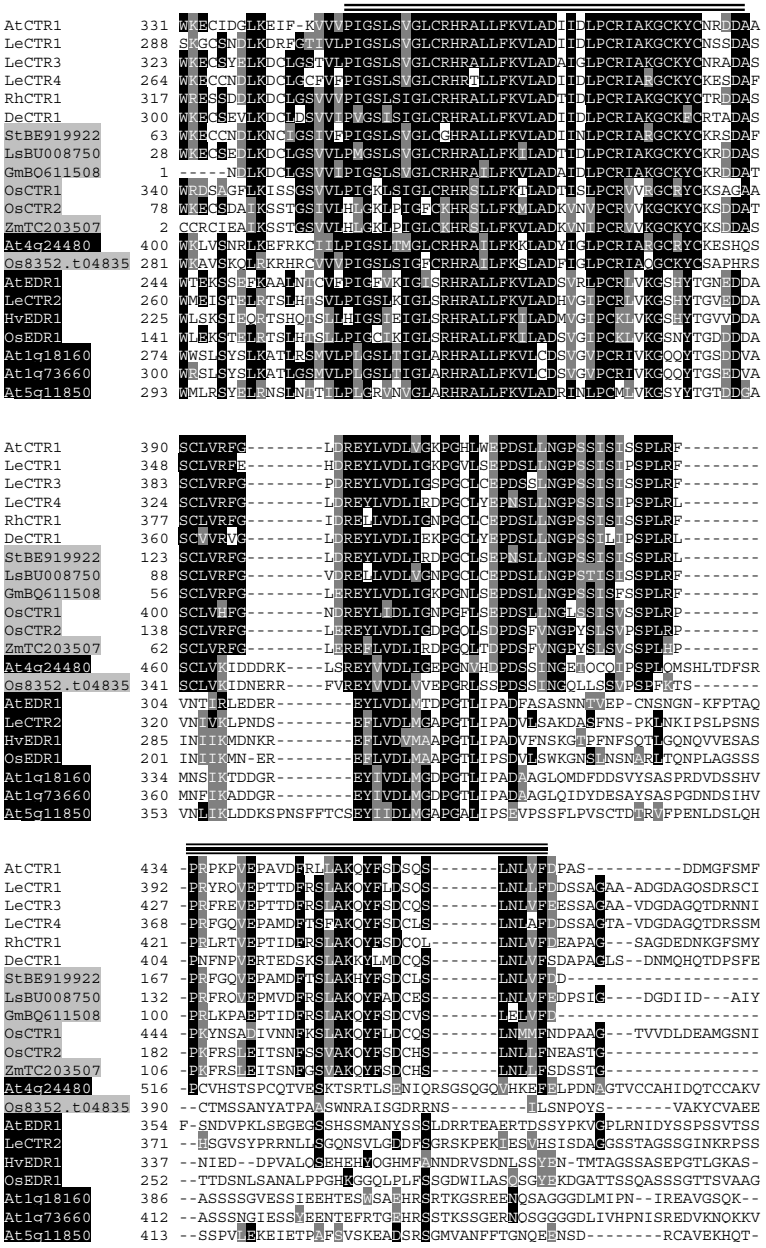

Figure 9. Conserved regions in the N-terminal domain are present in both AtCTR1 and putative CTR1-like amino acid sequences. Amino acid alignments were performed by ClustalX. Amino acid residues identical to the consensus sequence are shaded black while residues that are not identical but similar are shaded gray. Sequences retrieved from TIGR EST and genome database searches that spanned the CN domain and beyond were chosen for the alignment and are shown highlighted in gray. These putative *CTR1*-like sequences are preceded by a two-letter prefix to indicate the species of origin: St, *Solanum tuberosum* (potato), Ls, *Lactuca sativa* (lettuce), Gm, *Glycine max* (soybean), Os, *Oryza sativa* (rice), and Zm, *Zea mays* (maize). Sequences highlighted in black are both reported and putative MAPKKKs which belong to the same subfamily as AtCTR1 and are shown here to illustrate similarities and differences from *CTR1*-like sequences. The double line indicates the CN box (described by Huang *et al*., 2003). Downstream of the CN box, marked above with a triple line, is a region which appears to be conserved only in the *CTR1*-like sequences which we have designated the EC (ethylene CTR-specific) domain.

Discussion

Through isolation and functional characterization of three *LeCTR* cDNAs and corresponding genomic clones from tomato, we have provided experimental evidence of a multigene family of *CTR1*-like genes which are functionally able to participate in ethylene signal transduction. Isolation and structural analysis of the genomic clones of the tomato *CTR1*-like genes revealed that intron sizes were considerably larger than those found in *Arabidopsis CTR1* while the organization of introns/exons remained conserved. This is consistent with the observation that while the position of the introns was probably established before the divergence of tomato and *Arabidopsis*, differences exist between the two species in their rates of accumulation or loss of non-coding DNA (Ku *et al*., 2000). Exon size and position is well conserved between the tomato and *Arabidopsis* sequences with the notable exception of exon 6. The longest intron in the *Arabidopsis CTR1* sequence precedes exon 6 and was found to be spliced at reduced efficiency in the mRNA population (Kieber *et al*., 1993). Structural comparison of the tomato *CTR* genomic sequences revealed that exon 6 was interrupted by an intron in different locations in both *LeCTR3* and *LeCTR4sv1* coding sequences. It has been well documented that a common form of alternative splicing in plants is intron retention and presumably reflects poor recognition of the intron (Brown and Simpson, 1998). This may be the case for the *LeCTR1* and *LeCTR4*/*LeCTR4sv2* transcripts. While no intron is spliced out, consensus acceptor sites and donor sites are present. Of note is the fact that if the *LeCTR3* intron were read through in frame, several stop codons would be encountered which would render the protein non-functional. In the case of *LeCTR4sv1*, when the intron is spliced, a stop codon is brought into frame rendering the predicted protein non-functional, explaining the lack of complementation of the *ctr1-8* mutant for this construct. Further, the identification of two *LeCTR4* splice variants each differing only in the processing of this same intron permits speculation that splicing in the junction region which connects the N-terminal domain to the kinase domain could serve in autoregulation or pathway control as a trans-dominant inhibitor. In such a scenario, it would be possible that each *LeCTR* transcript could have splice variants that differ in the processing of this intron. This phenomenon has been previously shown to occur in broccoli, rice and wheat mRNA transcripts (reviewed by Brown and Simpson, 1998).

Attempts to complement the *Arabidopsis ctr1-8* mutation with three different tomato *CTR1* genes suggest all encode functional CTR1 proteins *in vivo*. Specifically, we have shown that all three genes have similar percent predicted amino acid identity to AtCTR1 (Table 1), all are more similar to *AtCTR1* than any other genes in the *Arabidopsis* genome (Figure 2) and when expressed in the *ctr1-8* mutant under the direction of the CaMV 35S promoter each resulted in partial to full complementation of mutant seedling (Figure 4) and mature plant phenotypes (Figures 5 and 6). While RNAi of each *LeCTR* gene is in progress in our lab, it is noteworthy that virus-induced gene silencing (VIGS) of the *LeCTR1* gene resulted in constitutive ethylene-response phenotypes in tomato (Liu *et al*., 2002).

The *LeCTR* gene family is differentially regulated by ethylene and during stages of development marked by increased ethylene biosynthesis. Similarly, ethylene receptors are encoded by a multi-gene family, differentially regulated by ethylene, and function to negatively regulate ethylene responses in both *Arabidopsis* and tomato (Hua and Meyerowitz, 1998; Lashbrook *et al*., 1998; Tieman and Klee, 1999). Somewhat paradoxical is the notion that expression of a negative regulator of ethylene response would increase in response to ethylene. This phenomenon may serve as a mechanism to modulate sensitivity to ethylene to provide the range of responses under various conditions/tissues observed for ethylene (Hua and Meyerowitz, 1998; Tieman *et al*., 2000; Klee, 2002). When ethylene is present it binds to the receptors to inhibit their biochemical activity, causing CTR1 to become inactive and unable to repress downstream responses leading to ethylene associated phenotypes (Huang *et al*., 2003). The ratio of receptors encoded by different family members in a particular cell type might influence the dose–response relationships which can vary for different tissues and responses (Bleecker, 1999). In apparent contrast to *Arabidopsis*, modulation of said ratio in tomato occurs at the levels of both receptors and CTRs, while only receptors respond transcriptionally to ethylene in *Arabidopsis*. The

combination of a larger repertoire of inducible *CTR* genes, in concert with an apparently greater range of inducibility of ethylene receptors in tomato as compared to *Arabidopsis*, may represent an adaptation to promote important biological functions dependent upon ethylene in the *Solanaceae*. In this regard it will be interesting to determine whether or not specific tomato CTRs will interact with specific tomato receptors. For example, one might predict that LeCTR1, which is inducible in ripening fruit, might interact *in vivo*, and possibly specifically, with the predominant fruit ethylene receptors Nr and LeETR4.

While *AtCTR1* is a part of the large *MAPKKK* gene family in *Arabidopsis*, it is curious why there is only one gene encoding CTR1 function in *Arabidopsis* while there are two *CTR1*-like sequences in its close relative *B. oleracea*. Additionally, there seemingly exists a small family in tomato, potato, lettuce, soybean, and rice. It will be interesting to ascertain whether or not multiple *CTR1*-like genes is a reflection of multiple MAP kinase cascades capable of participation in ethylene responses.

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